Comp. Biochem. Physiol. Vol. 94B, No. 1, pp. 49–51, 1989
Printed in Great Britain

0305-0491/89 $3.00 + 0.00
Pergamon Press plc

DEVELOPMENT OF AN AVIAN CALCITONIN RADIOIMMUNOASSAY USING SYNTHETIC CHICKEN CALCITONIN AS IMMUNOGEN

J. P. McMURTRY and N. C. STEELE

USDA-Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

(*Received* 9 *January* 1989)

Abstract—1. A sequential double antibody radioimmunoassay (RIA) has been developed using synthetic chicken calcitonin (CT) as antigen, tracer and standard.

2. The immunoassay has a minimum detection limit of 0.5 ng and effective dose (ED_{50}) of 7 ng. Serial dilutions of chicken and turkey plasma were parallel to serial dilutions of CT standard. Extracts of chicken and turkey ultimobranchial glands caused parallel displacement of tracer similar to synthetic CT.

3. Primary antisera (anti-chicken CT) was raised in guinea pigs immunized with RIBI; animals treated with Freund's complete adjuvant failed to respond.

4. Chicken CT was determined to have a half-life of 60 sec in the turkey hen. Development of a homologous RIA for avian CT will allow studies to elucidate the role of this hormone in birds.

INTRODUCTION

Calcitonin (CT) is a 32 amino acid polypeptide produced by the ultimobranchial glands in lower vertebrates and by the C cells of the thyroid gland in mammals (Copp, 1970). Homologous radioimmunoassays (RIA) have been developed for the rat (Burford *et al.*, 1975), human (Deftos, 1971), and salmon (Deftos *et al.*, 1974). Heterologus RIAs have been reported for bovine CT (Deftos *et al.*, 1979), ovine (Forslund and Stridsberg, 1980), and chicken (Cutler *et al.*, 1974; Lasmoles *et al.*, 1983).

Recently, the structure for chicken CT, derived from the nucleotide sequence of the cDNA, has been reported (Lasmoles *et al.*, 1985). In this paper the development of a homologous RIA for avian CT, using synthetic chicken CT as immunogen, tracer and standard is reported.

MATERIALS AND METHODS

Synthetic chicken CT was purchased from Peninsula Laboratories, Inc., Belmont, CA. Lot analysis information provided indicated a purity greater than 95%, as determined by high resolution HPLC and amino acid analysis. This material was used for all immunizations, tracer preparation and as standard.

Two adjuvants, Freund's complete (Sigma Chemical Co.) and Ribi Adjuvant System (RAS) (RIBI, Hamilton, MT) were utilized to generate antibodies in female guinea pigs. In both systems, synthetic chicken CT was dissolved in sterile saline to concentration of 200 μg/ml. To this was added an equal volume of either Freund's complete or RAS and thoroughly mixed prior to injection. Multiple site, intradermal injections were conducted at 4 week intervals. Guinea pigs were bled at two week intervals and the sera tested for the presence of anti-CT antibodies.

Synthetic chicken CT was iodinated by a modification of the chloramine T method (Greenwood *et al.*, 1963). Five μg of the peptide dissolved in 25 μl 0.3 M phosphate buffer, pH 7.4 was reacted with 0.8 mCi sodium iodide-I-125 (100 mCi/ml, New England Nuclear), plus 16 μg chloramine T for 60 sec. The reaction was terminated with 48 μg sodium metabisulfite, dissolved in 0.05 M sodium phosphate, then transferred in 500 μl elution buffer to a Sephadex G-50 column (0.5 × 50 cm) for chromatography (elution buffer = 0.05 M sodium phosphate, pH 7.4). Only those fractions on the ascending part of the protein peak were found to bind to the anti-chicken CT antisera. Using this method, a specific activity of 50 μCi/μg is routinely obtained. The tracer was found to be stable for 7 weeks when stored at 4°C and diluted in RIA buffer containing Trasylol (Mobay Chemical Co., New York).

A sequential RIA was conducted by adding 100 μl of guinea pig antiserum GP # 37 (at a working dilution of 1:4000) to tubes containing 100 μl RIA buffer (0.05 M sodium phosphate/0.05 M EDTA/1% bovine serum albumin/4000 U/mol Trasylol, pH 7.5) and 200 μl standard peptides diluted in RIA buffer. Duplicate standard hormone and unknown sample tubes were first incubated with primary antibody for 72 hr. Thereafter, 8000 cpm tracer in 100 μl RIA buffer were added and the incubation continued for 24 hr. To separate bound and free tracer, 100 μl of 1:200 normal guinea pig serum and 200 μl 1:10 sheep anti-guinea pig gamma globulin were added and the tubes incubated for an additional 72 hr. All incubations were conducted at 4°C. The precipitated antibody complex was sedimented by centrifugation. Intraassay and interassay coefficients of variation (based on a plasma pool) were calculated to be 2.3% and 9.9% respectively.

Individual ultimobranchial glands were obtained from adult chickens and turkeys and homogenized separately in 1.0 N HCl (1:10 w/v) in an ice bath. The homogenate was centrifuged, the supernatant collected and pH adjusted with ammonium hydroxide (8 N) for immunoassay. As needed, additional dilutions of the extract were made with RIA buffer. These preparations were used to test for parallelism in the RIA described previously.

Calcitonins and parathyroid hormone from other species were tested for cross-reactivity in the RIA. Human, eel, and salmon sequence CT were obtained from Sigma Chemical Co. Bovine PTH 1-34, was purchased from Peninsula Laboratories. Porcine CT (K 7001470) was kindly provided by J. Pento, Univ. of Oklahoma.

The metabolism ($T_{1/2}$) of chicken CT was determined in the same manner as previously described for chicken insulin (McMurtry *et al.*, 1987). Briefly, four Large White turkey hens (30 weeks of age) were implanted with jugular cannula

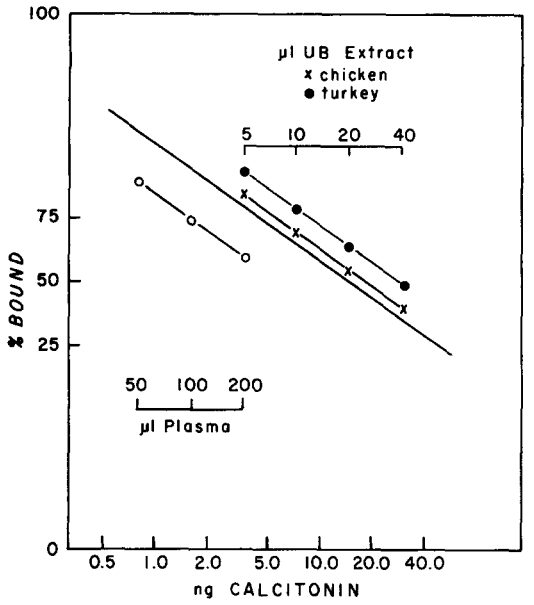

Fig. 1. Inhibition curves obtained with serial dilutions of synthetic chicken CT standard (——); extracts of ultimobranchial glands from the chicken (×——×) and turkey (●——●); turkey and chicken plasma (○——○) in a homologous immunoassay for chicken CT.

and infusion was conducted 7 days after cannulation. CT (100 µg, dissolved in 1 ml sterile saline) was injected as a bolus into a wing vein to avoid possible contamination of the sampling cannula. Heparinized samples were drawn at timed intervals (see Table 2) and the plasma stored in the presence of 100 KIU/ml Trasylol (Mobay Chemical Co., New York, NY). Plasma CT concentrations were determined by the previously described RIA.

RESULTS

Only those guinea pigs immunized using the RAS system were found to have anti-calcitonin antibodies. The antiserum (GP # 37) chosen for RIA development was of the greatest titer and provided the maximum sensitivity. Using synthetic chicken CT as standard and tracer, the double antibody immunoassay has a minimum detection limit of 0.5 and effective dose (ED_{50}) of 7 ng. Serial dilutions of chicken and turkey plasma were parallel to serial dilutions of CT standard (Fig. 1). Extracts of homogenized turkey and chicken ultimobranchial glands caused parallel displacement of tracer similar to synthetic CT (Fig. 1). The accuracy (recovery) of the RIA, over the range of concentrations tested, averaged 101% (Table 1). Several calcitonins (human, eel, pig and salmon) were tested for cross-reactivity in the RIA. Eel CT, which has a sequence similar to chicken CT (94%), (Lasmoles *et al.*, 1985), exhibited minimal cross-reaction (0.08%). None of the other peptides (salmon, human, porcine CT and bovine PTH) at concentrations to 10 µg displaced tracer. In addition, I-125 human CT (Immuno Nuclear Corp., Stillwater, MN), incubated in the presence of an excess of the guinea pig anti-chicken (GP # 37) antiserum, did not result in specific binding (1.1%). Half-life ($T_{1/2}$), based on the disappearance of synthetic CT, following a bolus injection into turkey hens, was calculated to be approximately 60 s (Table 2).

Table 1. Comparison of the recovery of chicken calcitonin from chicken and turkey plasma

Calcitonin added* (ng/tube)	Amount recovered Chicken plasma	Turkey plasma
0.65	0.67 + 0.01	0.69 + 0.02
1.30	1.33 + 0.03	1.28 + 0.04
2.60	2.65 + 0.07	2.61 + 0.06
5.20	5.07 + 0.15	5.10 + 0.11

*Chicken calcitonin dissolved in 5 µl saline was added in the above amounts to 195 µl of normal chicken and turkey plasma.
†Mean + SEM. $n = 5$.

Table 2. Plasma concentrations of chicken calcitonin injected into turkey hens for half-life determinations

Time (min)	Calcitonin (ng/ml)
0*	1.1†
2	22,139
4	12,678
6	6313
8	3041
10	1411
15	375
20	62
30	2.4
40	0.8
50	0.8
60	0.5
75	0.7
90	1.2
120	1.0
150	0.9
180	0.6

*Four pre-injection samples (−10, −5, −2, 0 min) were taken to establish a baseline calcitonin value.
†Mean calcitonin concentration; $n = 4$.

DISCUSSION

Unlike mammals, the functional significance of calcitonin (CT) in aves remains an enigma (Copp, 1976). Birds are a species in which one might expect to find calcium metabolism being exquisitely regulated, considering the high plasma calcium levels and the various demands on this system during different physiological states, e.g. bone growth, egg shell calcification. One of the reasons for a lack of understanding has been the absence of avian CT in suitable quantities for biochemical and physiological studies. With the structure of chicken CT being elucidated (Lasmoles *et al.*, 1985), this has facilitated the chemical synthesis of this hormone.

Heterologous RIAs for chicken CT have utilized synthetic salmon calcitonin as the source of immunogen, tracer and standard (Cutler *et al.*, 1974; Lasmoles *et al.*, 1983). A major concern is that many animals must be immunized to obtained an antisera that provides sufficient cross-reactivity and sensitivity to measure avian CT. As previously noted for other CT immunoassays, there is often little cross-reactivity among the various species of calcitonins (Deftos *et al.*, 1979).

In this paper we describe the development of a homologous RIA for chicken CT. The antiserum is highly specific and can be used to measure CT in other avian species (turkey). We have no explanation as to why only those guinea pigs immunized with the

RAS system produced antibodies, whereas those injected with Freund's adjuvant failed to respond.

In mammals CT has a half-life of 2 to 5 min (Lee *et al.*, 1969; Cooper *et al.*, 1971). Our study indicates that a shorter $T_{1/2}$ (60 sec) may exist in birds. This may explain in part the report that CT may not be a hypocalcemic factor in birds (Gonnerman *et al.*, 1972; Copp, 1976).

Acknowledgements—The authors wish to thank D. Brocht and W. Tsark for their assistance in these studies. Mention of a trade name, proprietary product or vendor does not constitute a guarantee or warranty of the product by USDA or imply exclusion of other products or vendors that may also be suitable.

REFERENCES

Burford H., Ontjes D., Cooper C., Perlow A. and Hirsch P. (1975) Purification, characterization and radioimmunoassay of thyrocalcitonin from rat thyroid glands. *Endocrinology* **96,** 340–348.

Cooper C., Deftos L. and Potts J., Jr. (1971) Direct measurement of *in vivo* secretion of porcine calcitonin by radioimmunoassay. *Endocrinology* **88,** 747–754.

Copp D. (1970) Endocrine regulation of calcium metabolism. *Ann. Rev. Physiol.* **32,** 61–85.

Copp D. (1976) Comparative endocrinology of calcitonin. In *Handbook of Endocrinology*, Vol. 7, pp. 431–432. Am. Physiol. Soc., Washington, D.C.

Cutler G., Habener J., Dee P. and Potts J., Jr. (1974) Radioimmunoassay for chicken calcitonin. *FEBS* **38,** 209–212.

Deftos L. (1971) Immunoassay for human calcitonin. I. Method. Metabolism **20,** 1122–1128.

Deftos L., Watts E., Copp D. and Potts J., Jr. (1974) A radioimmunoassay for salmon calcitonin. *Endocrinology* **94,** 155–160.

Deftos L., Habener J., Mayer G., Bury A. and Potts J., Jr. (1979) Radioimmunoassay for bovine calcitonin. *J. Lab. Clin. Med.* **79,** 480–490.

Forslund K. and Stridsberg M. (1980) Evaluation of a radioimmuoassay technique measuring calcitonin in the bovine, ovine and porcine species. *Acta Vet. Scand.* **21,** 155–170.

Gonnerman W., Breitenbach R., Erfling W. and Anast C. (1972) An analysis of ultimobranchial gland function in the chicken. *Endocrinology* **91,** 1423–1429.

Greenwood F., Hunter W. and Glover J. (1963) The preparation of 131-I-labelled human growth hormone of high specific activity. *Biochem. J.* **89,** 114–123.

Lasmoles F., Desplan C., Segond N., Jullienne A., Treilhou-Lahille Milhaud G. and Moukhtar M. (1983) Cell free translation of chicken calcitonin messenger RNA. *FEBS* **157,** 100–104.

Lasmoles F., Jullienne A., Desplan C., Milhaud G. and Moukhtar M. (1985) Structure of chicken calcitonin predicted by partial nucleotide sequence of its precursor. *FEBS* **180,** 113–116.

Lee M., Deftos L. and Potts J., Jr. (1969) Control of secretion of thyrocalcitonin in the rabbit as evaluated by radioimmunoassay. *Endocrinology* **84,** 36–40.

McMurtry J., Rosebrough R. and Steele N. (1987) Insulin metabolism and its effect on blood electrolytes and glucose in the turkey hen. *Comp. Biochem. Physiol.* **86A,** 309–313.